International Journal of Cardiology 177 (2014) 409–417

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Remote ischemic preconditioning preserves mitochondrial function and activates pro-survival protein kinase Akt in the left ventricle during cardiac surgery: A randomized trial☆

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ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 24 August 2014
Accepted 15 September 2014
Available online 14 October 2014

Keywords:
Cardiopulmonary bypass
Mitochondria
Proteins
Myocardium
Preconditioning

ABSTRACT

Background: Understanding the intracellular mechanisms induced by remote ischemic preconditioning (RIPC) in the human left ventricle opens new possibilities for development of pharmacological cardioprotection against ischemia and reperfusion injury. In this study we investigated the effects of RIPC on mitochondrial function, activation of pro-survival protein kinase Akt and microRNA expression in left ventricular biopsies from patients undergoing coronary artery bypass surgery (CABG).

Methods: Sixty patients were randomized to control (n = 30) or RIPC (n = 30). A blood pressure cuff was applied to the arm of all patients preoperatively. The cuff remained deflated in control group, whereas RIPC was performed by 3 cycles of cuff inflation to 200 mm Hg for 5 min, separated by 5 min deflation intervals. Left ventricular biopsies were obtained before and 15 min after aortic declamping. The primary outcome was mitochondrial respiration measured in situ. Secondary outcomes were activation of protein kinase Akt, assessed by western immunoblotting, and expression of microRNAs assessed by array and real-time polymerase chain reaction.

Results: Mitochondrial respiration was preserved during surgery in patients receiving RIPC (+0.2 μmol O_2/min/g, p = 0.69), and reduced by 15% in controls (−1.5 μmol O_2/min/g, p = 0.02). Furthermore, RIPC activated protein kinase Akt before aortic clamping (difference from control +43.3%, p = 0.04), followed by increased phosphorylation of Akt substrates at reperfusion (+26.8%, p < 0.01). No differences were observed in microRNA expression.

Conclusions: RIPC preserves mitochondrial function and activates pro-survival protein kinase Akt in left ventricle of patients undergoing CABG. Modulation of mitochondrial function and Akt activation should be further explored as cardioprotective drug targets.

Clinical Trial Registration: http://www.clinicaltrials.gov, unique identifier: NCT01308138.

1. Introduction

Remote ischemic preconditioning (RIPC) has emerged as a promising strategy to reduce myocardial reperfusion injury after cardiac surgery [1]. RIPC involves exposing a tissue to brief, non-harmful periods of ischemia to induce protection against subsequent ischemic challenge in another organ. The cardioprotective effects of RIPC have been extensively investigated in animal models, revealing potential molecular targets for pharmacological cardioprotection. Previous studies have shown that regulation of mitochondrial function and activation of pro-survival protein kinase Akt are involved in the cardioprotection induced by RIPC [2,3], and that these cellular mechanisms interact [4].

Conserved mitochondrial function is required for optimal cardiac function, since it directly influences physiological processes that are essential for cardiomyocyte survival and proper contractile activity,

Abbreviations: RIPC, remote ischemic preconditioning; CABG, coronary artery bypass graft; CPB, cardiopulmonary bypass; ACC, aortic cross-clamping; cTnT, cardiac troponin T; CK-MB, creatine kinase-MB; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; ADP, adenosine diphosphate; ACR, acceptor control ratio; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; AUC, area under the curve; ATP, adenosine triphosphate; qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

☆ Grant support: This work was supported by the Liaison Committee between the Central Norway Regional Health Authority and the Norwegian University of Science and Technology (NTNU); Unimed Innovation's Research Fund; Norwegian Council on Cardiovascular Disease; K.G. Jebsen Foundation; and Department of Circulation and Medical Imaging, NTNU. The funding sources had no role in conception, analysis, discussion or interpretation of the study.

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http://dx.doi.org/10.1016/j.ijcard.2014.09.206
0167-5273/

including maintenance of energy substrates (ATP), pH control and scavenging of reactive oxygen species [5]. Previous studies have shown that both local ischemic preconditioning and RIPC prevent impairment of mitochondrial respiration induced by ischemia in rat skeletal muscle [6], and that maintaining an optimal mitochondrial function plays an important role in protecting the heart against ischemia [3,7]. Moreover, mitochondrial damage has been unequivocally demonstrated as a trigger of apoptotic cardiomyocyte death [8,9]. Preclinical studies showed that local ischemic preconditioning and RIPC reduce ischemic cardiac damage by blocking apoptosis through activation of pro-survival protein kinase Akt [10]. Accordingly, inhibition of Akt signaling completely blocks the effects of RIPC in a porcine model [11], while targeted activation of Akt renders potent cardioprotection in vivo [12].

Experimental studies also reported a causal involvement of microRNAs (e.g. microRNAs 199a and 320) [13,14] in ischemia–reperfusion injury and mitochondrial physiology [15]. However, the effects of RIPC on left ventricular mitochondrial function and microRNA expression have never been explored in humans.

Despite robust preclinical evidence, intracellular mechanisms induced by RIPC in the human left ventricle are nearly unexplored. Therefore, we investigated the effects of RIPC on left ventricular mitochondrial function, microRNA expression and activation of protein kinase Akt in patients undergoing coronary artery bypass graft (CABG) surgery.

2. Methods

2.1. Study design and participants

This single-center, randomized, prospective, double-blinded study included sixty patients admitted for urgent or elective first-time on-pump CABG surgery at St. Olav's Hospital, Trondheim University Hospital, Norway. The study was conducted in 2011. Exclusion criteria were severe hepatic, renal or pulmonary disease, and peripheral vascular disease of the upper limbs. A database provided by the Unit for Applied Clinical Research at St. Olav's Hospital was used for randomization. Randomization was accomplished immediately before performing the procedure. Patients, surgeons and personnel (both in postoperative intensive care as well as in the laboratory) were blinded to group allocations until after completion of data collection and analyses. This investigation conforms to the principles outlined in the Declaration of Helsinki and was approved by the Regional Committee for Medical Research Ethics of Norway (REK 2010/461-9). Written informed consent was obtained from all patients prior to inclusion. The study is registered at www.clinicaltrials.gov, identification number NCT01308138.

Premedication in the form of acetaminophen and morphine-scopolamine was administered 1–3 h before surgery. Intravenous thiopental, fentanyl, propofol and cisatracurium were used for anesthesia, supplemented by the volatile anesthetic isoflurane, which was administered during pulmonary ventilation before and after cardiopulmonary bypass (CPB). CPB was conducted with a membrane oxygenator at mild hypothermia of 34 °C. Cold crystalloid or blood cardioplegia with standard St. Thomas' solution (Martindale Pharmaceuticals, United Kingdom) was given every ~20 min. Distal coronary anastomoses were constructed under aortic cross-clamping (ACC). All perioperative procedures were performed according to standard routines of the department, including presurgical preparations, anesthetics, drug administration, surgical technique and postoperative care.

2.2. Intervention

All patients included in the study had a blood-pressure cuff applied to the upper arm before induction of anesthesia. For patients randomized to RIPC ($n = 30$), the cuff was inflated to 200 mm Hg for 3 cycles of 5 min ischemia and 5 min reperfusion, after the induction of anesthesia. The cuff remained deflated for an equivalent period in control patients ($n = 30$), Harris J, Barnard M, Grundy E, (Fig. 1).

2.3. Biochemical markers

Blood samples were collected preoperatively (T1), 3 h after removal of ACC (T2), 6 h after removal of ACC (T3), as well as on the first postoperative day (T4). Analyses of circulating biochemical markers (creatinine; CRP, C-reactive protein; cTnT, cardiac troponin T; CK-MB, creatine kinase-MB; and pro-BNP, N-terminal pro-brain natriuretic peptide) were performed at the laboratory of St. Olav's Hospital according to standard procedures.

2.4. Ventricular biopsies

Left ventricular samples were obtained by use of a disposable automatic 16G biopsy needle (BioPince™ Full Core Biopsy Instrument) during CPB at two separate intraoperative time-points; the first before application of ACC (pre-ACC) and the second ~15 min after removal of ACC (post-ACC). One part of the biopsy was added to ice-cold storage solution for mitochondrial analyses, one part was immersed in RNAlater® (Ambion®) and one part was immediately snap-frozen in liquid nitrogen for protein analyses. The sample in RNAlater and the snap-frozen sample were maintained frozen at −80 °C until further processing.

2.5. Mitochondrial respiration in situ

Mitochondrial respiration was measured in situ, as described [16,17]. The tissue was continuously kept at 4 °C during preparatory procedures. The myocardium was kept in storage solution from harvest until membrane permeabilization with 50 μg/mL saponin for 30 min, followed by a rinse cycle of 10 min in pure storage solution and an additional 10 min in respiration solution. The storage solution contained (in mmol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole, 50 potassium-methanesulfonate (CH_3KO_3S), 5.7 Na_2ATP, and 15 phosphocreatine (PCr) (pH 7.1 at 22 °C). The respiration solution contained (in mmol/L) 2.77 CaK_2EGTA, 7.23 K_2EGTA, 1.38 $MgCl_2$ (1 mmol/L free Mg^{2+}), 20 taurine, 0.5 dithiothreitol (DTT), 20 imidazole (pH 7.1 at 22 °C), 90 potassium-methanesulfonate (CH_3KO_3S), 10 sodium-methanesulfonate (CH_3SO_3Na), 3 K_2HPO_4, 10 glutamate, 4 malate, and 2 mg/mL bovine serum albumin. Assessment of mitochondrial function was performed in 3 mL of respiration solution at 22 °C with a fluorinated ethylene propylene membrane on a Clark-type microcathode oxygen electrode (Strathkelvin Instruments, UK). Measurement of basal respiration rate (V_0) with glutamate and malate as substrates for respiratory Complex I was followed by addition of subsaturating amount (0.1 mmol/L) of adenosine diphosphate (ADP) (measuring V_{ADP}) and subsequently 20 mmol/L creatine (measuring $V_{creatine}$). Supplement of a saturating amount of ADP (2 mmol/L) allowed assessment of respiration rates involving the entire respiratory chain including Complexes I through IV with glutamate and malate as substrates, recording maximal respiration rate (V_{max}). Complex II substrate succinate (10 mmol/L) was added to assess $V_{succinate}$, after which supplement of Complex I inhibitor amytal (1 mmol/L amobarbital) allowed assessment of Complex II (V_{amytal}). Lastly, ascorbate (0.5 mmol/L) and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 0.5 mmol/L) were added to induce respiration of Complex IV ($V_{ascorbateTMPD}$). Respiratory rates are given as micromoles O_2 per minute per gram dry weight of left ventricular tissue (μmol O_2/min/g dw). The acceptor control ratio (ACR) was calculated from the ratio of V_{max}/V_0 to quantify the degree of coupling between oxidation and phosphorylation. Mitochondrial sensitivity to ADP was estimated by the ratio of V_{ADP}/V_{max}. Excess respiration of the cytochrome oxidase complex was quantified by the ratio V_{amytal}/V_{max}. Effect of creatine is given as percent increase in respiration rate after the addition of creatine (↑RR Cr). The apparent constant of Michaelis for ADP was estimated in the absence ($^{app}K_m^{(ADP-Cr)}$) and presence of creatine ($^{app}K_m^{(ADP+Cr)}$) [16].

2.6. MicroRNA expression

Random selection of pre-ACC and post-ACC ventricular samples from 10 patients in RIPC and 10 patients in control group was used for microRNA analyses. Samples were transferred on dry ice transportation-medium from St. Olav's Hospital, Trondheim University Hospital, Norway to Exiqon Services, Denmark (<24 h), where microRNA analyses were performed. RNA samples were reverse transcribed into complementary DNA in triplicate. Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, California) was used to evaluate RNA quality. Samples were labeled for microRNA array with miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark), and hybridization was performed on the miRCURY LNA™ microRNA Array (6th gen) (Exiqon, Denmark) including capture probes for all human miRs registered in the miRBASE 16.0. miRCURY LNA™ Universal RT miRNA PCR (Exiqon, Denmark) human pick and mix panel was used for microRNA quantitative reverse-transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed for microRNA-1, -125b-1*, -129*, -133a, -133b, -185, -191, -199a-3p, -199a-5p, -208b, -21, -210, -23a, -299-5p, -320a, -320b, -338-3p, -423-3p, -494, -525-5p, -630, -92a, and miR-943. Average values from three out of four preselected normalization assays were applied for normalization.

2.7. Western immunoblotting

Biopsies were kept stored at −80 °C and untouched until all samples were collected. All biopsies were prepared for analysis on the same day to guarantee identical handling procedures. Samples were homogenized in ice-cold RIPA buffer (150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0; Sigma-Aldrich, Germany) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, Germany). Lysates were centrifuged (12,000 *g*, 4 °C, 15 min) to remove pelleted debris. Total protein concentration in the lysates was measured in triplicate by the BCA method (Thermo Scientific, Norway). Total protein yield did not differ between control and RIPC groups. Sample loading buffer supplemented with reducing agent was added to the lysates and samples were heated at 95 °C for 10 min in a heating block for protein denaturation. Ten micrograms of total proteins was loaded into polyacrylamide Bis-Tris gels (4–20% or 10% acrylamide, Bolt Precast Gels, Life Technologies, Norway) and subjected to electrophoresis at 165 V. Gels were removed from cassettes and proteins were blotted to nitrocellulose membranes by dry transfer (P0 protocol with iBlot system, Life Technologies, Norway). Effective and even transfer was verified by Ponceau staining (Sigma-Aldrich, Norway) of the membrane (5 min at room temperature). Membranes were blocked for 1 h at room temperature (5% BSA in PBS plus 0.05% Tween-20), followed by incubation with primary antibody (1:1000 dilution in blocking buffer, overnight at 4 °C). Primary antibodies against total Akt, total P70-S6k, total GSK3β, total ribosomal S6 protein, phosphorylated Akt (serine 473), phosphorylated targets of Akt, phosphorylated GSK3-beta (serine 9) and phosphorylated ribosomal S6 protein (Serine 240/244) were

used. Information regarding antibodies is available in Supplemental Table 1. All membranes were also incubated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, which was adopted as loading control. We tested all antibodies in advance to verify specificity (absence of unspecific binding). Membranes were washed three times with PBS without BSA (5 min each, room temperature) and incubated for 1 h with fluorescent-labeled secondary antibodies (anti-Rabbit at 1:20,000 or anti-Mouse at 1:10,000) (LICOR Biosciences, UK). Membranes were washed again and imaged by fluorescence scanner (Odyssey system, LI-COR Biosciences, UK). Signals were objectively quantified. Images were analyzed without any modification from the original scanning, and sample pictures were converted to gray-scale for illustration purposes in the manuscript. Phosphorylated protein expression was normalized to total protein expression. Mean values of control group before ACC were arbitrarily set as 100% and data are presented as relative percentage difference from the control group (pre-ACC).

2.8. Statistical analysis

Sample size was estimated from previous assessment of the effect of RIPC on troponin release after CABG, requiring a minimum of 27 patients per group to maintain a statistical power of 80% [1]. SPSS 19.0 for Mac (IBM SPSS Statistics, Chicago, Illinois) was applied for statistical calculations. For continuous variables, unpaired or paired Student's t-test was used to assess between-group and within-group (pre-ACC vs. post-ACC) differences, respectively. For categorical variables Pearson's chi squared test and Fisher's exact test were used. A two-tailed p-value < 0.05 was considered significant. Bonferroni correction was applied to microRNA analyses. GraphPad Prism 5 (GraphPad Software Inc., USA) was used for graphical presentation of data.

3. Results

A diagram of the inclusion process is presented in Fig. 2. Sixty patients were included in the study and there was no 30-day mortality. RIPC and control groups were comparable with respect to anesthetics, intraoperative parameters and patient characteristics. Patient characteristics, previous medication and operative parameters are provided in Table 1. There was no significant difference in length of stay in postoperative intensive care.

3.1. Biochemical markers

Laboratory values were similar between groups pre- and postoperatively, including creatinine and C-reactive protein. No differences were observed in postoperative levels of cTnT, CK-MB or pro-BNP, for peak values or area under the curve (AUC) (Fig. 3).

Studies have suggested that the effects of ischemic preconditioning might be hindered by diabetes [18,19]. For this reason we conducted a subanalysis with data from nondiabetic patients only and observed that AUC (24 h) for cTnT and CK-MB were significantly lower in RIPC ($n = 23$ nondiabetic patients) than control ($n = 24$ nondiabetic patients) group (Supplemental Fig. 1). Patient characteristics, medication and intraoperative parameters of the nondiabetic subgroups are presented in Supplemental Table 2.

3.2. Mitochondrial respiration

All mitochondrial respiratory rates within the control group were reduced post-ACC compared to pre-ACC, including a significant reduction of V_{max} by an average of −15% (pre-ACC 10.2 ± 3.9 vs. post-ACC 8.7 ± 2.5 μmol O_2/min/g, $p = 0.02$, Fig. 4A). On the other hand, all respiration rates within RIPC were preserved throughout surgery, including V_{max} (pre-ACC 9.8 ± 3.9 vs. post-ACC 10.0 ± 3.0 μmol O_2/min/g, $p = 0.69$, Fig. 4B). The protective effect of RIPC on mitochondrial function was maintained when the subset of nondiabetic patients was analyzed (Supplemental Fig. 2).

No significant alterations were observed throughout surgery regarding ACR, ↑RR Cr, ${}^{app}K_m^{(ADP\text{-}Cr)}$, ${}^{app}K_m^{(ADP+Cr)}$, V_{ADP}/V_{max} or V_{amytal}/V_{max} in either group (Table 2).

3.3. MicroRNA expression

No significant differences were observed in left ventricular microRNA expression from pre-ACC vs. post-ACC within RIPC or control, or between RIPC vs. control.

3.4. Protein kinase Akt activation

Protein kinase Akt is activated by phosphorylation. Therefore, we assessed both total (non-phosphorylated) and phosphorylated forms of Akt. We also assessed phosphorylation of Akt-specific targets, as well as downstream players of Akt signaling that have been altered by RIPC in animal models.

When total protein expression of Akt, P70-S6k, GSK3β and ribosomal S6 protein was analyzed, no differences were observed between groups or between pre-ACC and post-ACC biopsies (Fig. 5, panels A–D). Abundance of phosphorylated Akt at serine-473 was 43.3% greater (95% CI for mean difference, +1.7 to +84.9%; $p < 0.05$) in RIPC than control (Fig. 5E), demonstrating early Akt activation by RIPC before the onset of ischemia (pre-ACC). Phosphorylation status of Akt in the RIPC group was maintained at reperfusion. Phosphorylation of Akt substrates increased 26.8% from pre- to post-ACC only in the RIPC group (95% CI, +10.5 to +43.2%; $p < 0.01$) and was higher in RIPC than control at reperfusion (post-ACC; $p < 0.05$) (Fig. 5F). Phosphorylation of GSK3β did not differ between groups before ACC or at reperfusion (Fig. 5G). Increased phosphorylation of ribosomal S6 protein (target of P70-S6k) was observed from pre- to post-ACC in both groups, without differences between RIPC and control (Fig. 5H).

In the subanalysis of nondiabetic patients, the phosphorylation level of Akt was higher after RIPC compared with control before ACC, and was further augmented at reperfusion (Supplemental Fig. 3). In this subanalysis we also observed a trend towards more abundant ($p = 0.07$) levels of phosphorylated GSK3β in RIPC than control group post-ACC (Supplemental Fig. 3).

4. Discussion

This is the first study to demonstrate that RIPC preserves mitochondrial respiration in the left ventricle of patients undergoing cardiac surgery. Maintaining adequate mitochondrial respiratory capacity is fundamental for limiting the extent of damage caused by cardiac ischemia–reperfusion [5,20]. As mitochondria contribute to approximately 90% of the ~30 kg ATP needed for optimal cardiac function on an average day [21,22], a 15% reduction of maximal mitochondrial respiratory capacity poses a substantial energetic disadvantage to the left ventricle, both during and after cardiac ischemia. Our results add support to previous studies demonstrating that ischemic preconditioning applied directly to the heart conserved myocardial ATP levels in patients during cardiac surgery [23].

Our data indicate that RIPC protected several complexes in the electron transport chain of left ventricular mitochondria, as we observed preserved respiratory capacity in Complexes I, II and IV after RIPC. Research on mitochondrial function in human myocardium is sparse, but a recent study demonstrated a more specific defect in Complex II of the respiratory chain in human ventricular tissue exposed to chronic ischemia [24]. A progressive defect in respiratory Complex I has been detected in human myocardium from patients at early stages of heart failure [25]. Studies of mitochondrial respiration in rodent skeletal muscle have implicated both a specific ischemia-induced dysfunction and subsequent protection by ischemic preconditioning localized to Complexes I and II in the aftermath of acute ischemia [6], whereas others observed a general malfunction of all respiratory complexes after ischemia–reperfusion [26]. The inconsistency in the precise localization of the respiratory alterations might be due to differences in species, tissue or severity of the ischemic challenge, which complicates a direct comparison with our results. RIPC preserved mitochondrial capacity in all steps of the protocol used in our study, indicating that the protection was not limited to specific mitochondrial complexes.

ACR remained similar from pre-ACC to post-ACC in samples from both groups, indicating that coupling between oxidation and phosphorylation was not altered by cardiac ischemia–reperfusion,

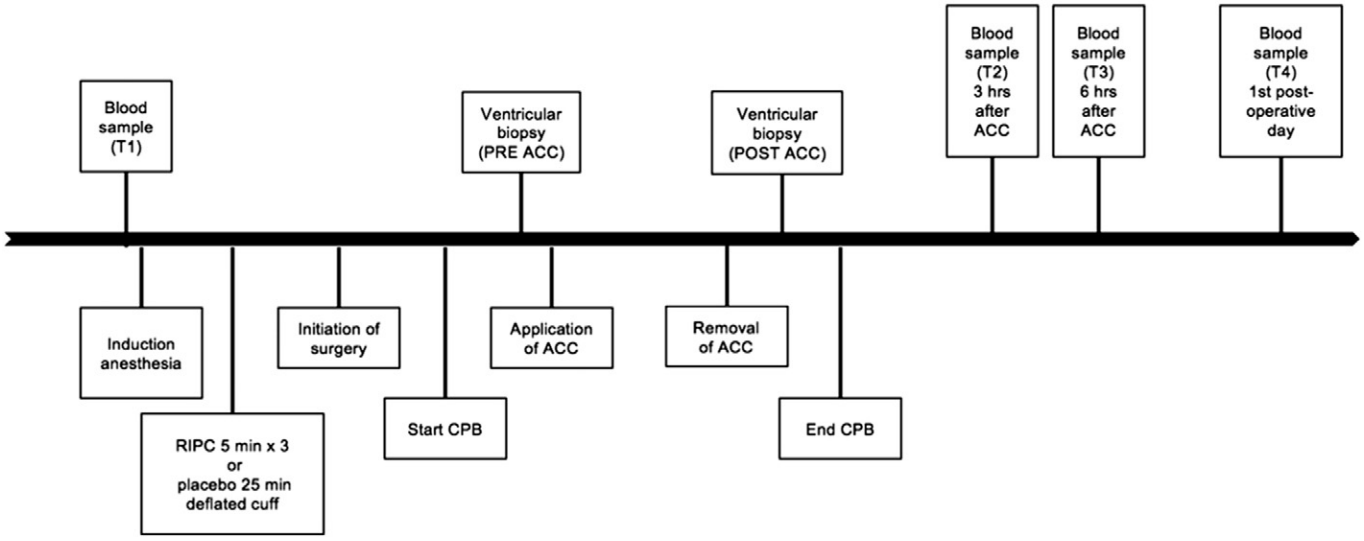

Fig. 1. Timeline illustrating sequence of procedures and sampling.

despite the reduced respiration rates observed in control samples after ACC. This result indicates that the reduced oxygen respiration observed in control group post-ACC resulted in lower amounts of ATP produced by mitochondria. Furthermore, values for ${}^{app}K_m^{(ADP+Cr)}$ and ${}^{app}K_m^{(ADP-Cr)}$ did not change significantly throughout surgery in either group indicating that the sensitivity to ADP was maintained at a stable level, and that mitochondrial creatine-kinase system was not affected by cardiac ischemia–reperfusion during surgery.

Increasing evidence demonstrates that energy metabolism and cell survival are not isolated phenomena, but interlaced mechanisms with reciprocate feedback [27], where mitochondria play a crucial role. Besides producing ATP for cardiac work, mitochondria are pivotal regulators of apoptotic signaling in cardiac myocytes. Dysfunctional mitochondria release cytochrome C to the cytosol, thereby activating apoptotic proteins (caspases) and causing myocardial necrosis in humans [9]. Such mechanism is blocked by RIPC in animal models through activation of protein kinase Akt [10]. Our study demonstrates that RIPC led to increased Akt phosphorylation before ACC when compared to the control group, suggesting an early activation of the kinase. Given this observation, we also used an antibody that recognizes proteins phosphorylated at motifs specifically targeted by Akt. Phosphorylation of Akt targets increased between pre- and post-ACC within the RIPC group only, reaching values significantly higher than the control group post-ACC. These observations suggest a delay between the time Akt is phosphorylated and the time that the kinase effectively acts (i.e. phosphorylates its targets). This phenomenon is unexplored in the context of ischemia–reperfusion, but might be important because phosphorylation of Akt is the trigger for kinase action, while not the final event to stop cell death. After activation by phosphorylation, Akt directly inhibits apoptotic proteins, thereby blocking the signaling leading to cell death [28]. Therefore, activation of Akt before ischemia might benefit myocardial cell survival during cardiac surgery.

The lack of alterations in microRNA expression contradicts a previously suggested association with mitochondrial function [15]. However, unchanged microRNA expression may be explained by the short interval between collection of pre- and post-ACC biopsies (35–70 min) in our study [29]. There were obvious obstacles impeding collection of

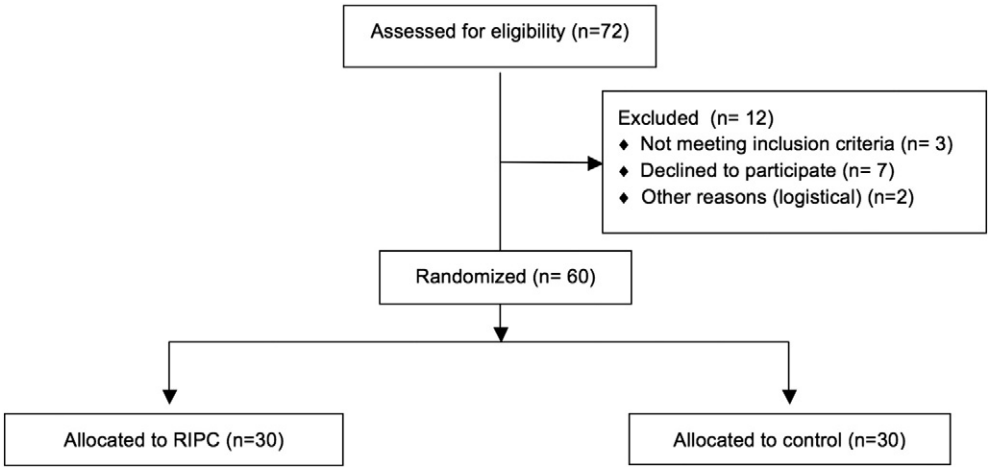

Fig. 2. CONSORT diagram of inclusion process.

Table 1
Clinical data.

	Control (n = 30)	RIPC (n = 30)	p-Value
Baseline characteristics			
Gender, men/women, n	23/7	27/3	0.17
Age, years	68 ± 8	64 ± 9	0.09
Body mass index, kg/m^2	28 ± 4	29 ± 8	0.48
ASA score	3.6 ± 0.6	3.4 ± 0.5	0.23
Euroscore II, %	1.31 ± 0.9	1.35 ± 1.2	0.90
Urgency, elective/urgent[a], n	13/17	15/15	0.61
Left ventricular ejection fraction, %	53 ± 7	52 ± 8	0.65
Creatinine clearance, mL/min	90 ± 25	102 ± 41	0.19
Risk factors and comorbidities			
Chronic obstructive pulmonary disease	2	3	>0.99
Current smoker	8	8	>0.99
History of smoking	21	20	0.78
Diabetes mellitus	6	7	0.75
History of atrial fibrillation	2	1	>0.99
Hypertension	15	12	0.44
Peripheral arterial disease	1	1	>0.99
Previous cerebral insult	0	3	0.24
Previous myocardial infarction	14	19	0.19
Previous PCI	5	5	>0.99
Unstable angina < 3 days preoperative	3	0	0.24
Pharmacotherapy			
ACE-inhibitor or ARB	11	14	0.43
Aspirin	29	28	>0.99
Beta blocker	24	25	0.74
Calcium channel blocker	9	1	0.01
Clopidogrel	11	13	0.60
Dipyridamole	0	2	0.49
Diuretics	4	7	0.32
Glibenclamide	1	0	>0.99
Insulin	1	2	>0.99
Lipid-lowering agent	27	25	0.71
Metformin	4	3	>0.99
Organic nitrates	6	3	0.47
Sulfonylurea	1	1	>0.99
Warfarin	2	1	>0.99
Intraoperative data			
Aortic cross-clamping, min	42 ± 11	41 ± 11	0.80
Cardiopulmonary bypass, min	76 ± 23	71 ± 14	0.31
Cardioplegia, mL	1000 ± 284	911 ± 257	0.21
Cardioplegia, cold blood/crystalloid, n	1/29	2/28	>0.99
Distal coronary graft anastomoses, n	3.3 ± 0.7	3.5 ± 1.0	0.31

Data are presented as mean ± SD or number. ASA-score, preoperative physical assessment score developed by the American Society of Anesthesiology; PCI, percutaneous coronary intervention; ACE, angiotensin converting enzyme; ARB, angiotensin-II-receptor blocker.

[a] Patients with a need for surgery during the current hospital admission (as defined in EuroSCORE II) [41].

left ventricular biopsies postoperatively, and therefore we cannot exclude the possibility that microRNAs play a role in cardiac responses to ischemia–reperfusion or RIPC at later time-points.

In this study we did not perform gain- or loss-of-function interventions to verify whether mitochondrial preservation and Akt activation are causes or consequences of RIPC-mediated cardioprotection in humans. Interventional disruption of mitochondrial function or pro-survival signaling is not, and will probably never be, an option in human studies, therefore, we must rely on experimental reports to extrapolate our results. In this regard, several studies demonstrated that cardiac ischemic preconditioning is abolished by blockage of mitochondrial ATP-dependent potassium channels due to collapse of mitochondrial membrane potential and oxidative phosphorylation (reviewed by Sato and Marban) [30]. In addition, a clinical study demonstrated that inhibition of mitochondrial transition pore opening by intravenous cyclosporine A (2.5 mg/kg body weight) reduces ischemic damage of the human heart [31]. Similarly, cardiac protection by RIPC is completely abolished in pigs pretreated with an inhibitor of

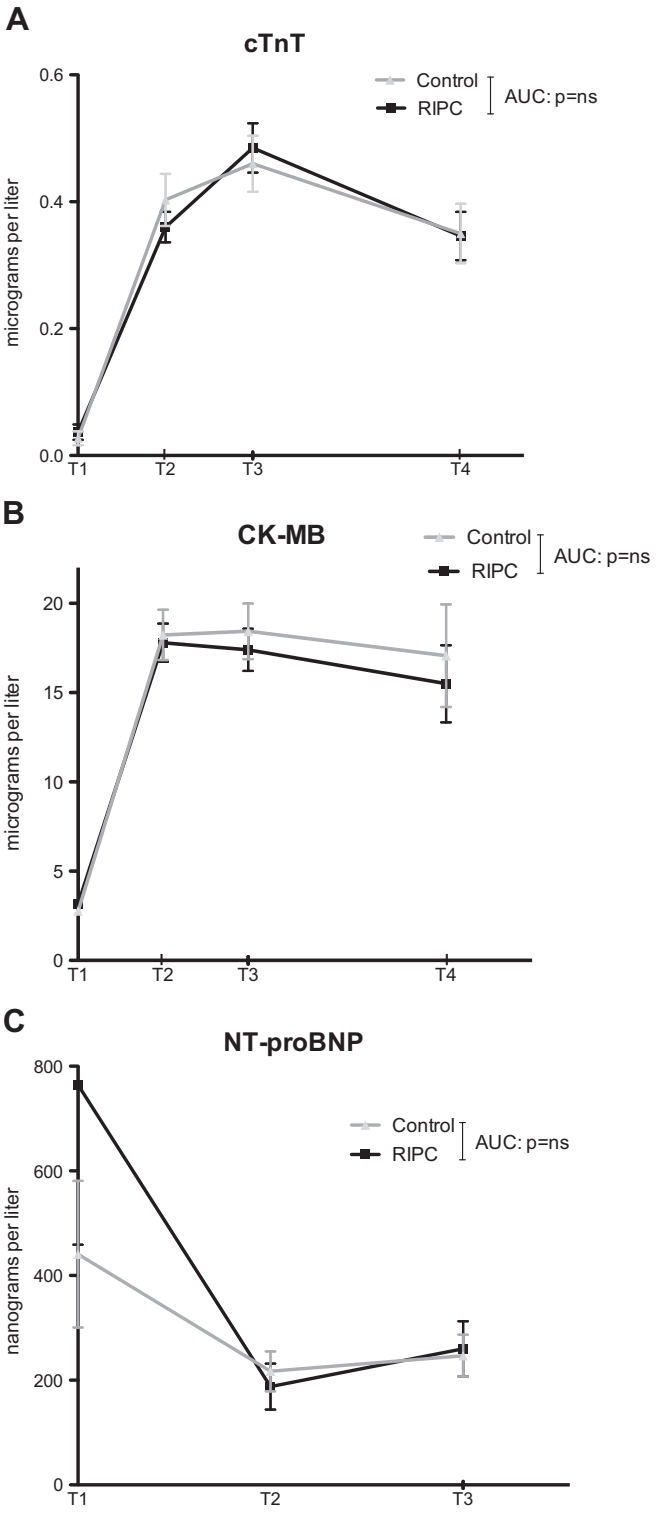

Fig. 3. Biochemical markers. Circulating (A) cTnT, (B) CK-MB and (B) NT-proBNP in control (n = 30) vs. RIPC (n = 30) group. Values are given as mean ± standard error (SEM). T1, preoperative; T2, 3 h after ACC; T3, 6 h after ACC; T4, 1-day postoperative.

Akt signaling [11], while transgenic activation of Akt protects the mitochondria [4], inhibits apoptosis and reduces ischemic damage after myocardial infarction in rodents [12]. Therefore, overwhelming evidence supports the rationale that pharmacological activation of Akt is an attractive strategy to preserve mitochondrial function and reduce ischemic cardiac damage. However, a selective Akt agonist has not been described in the literature, but should be pursued.

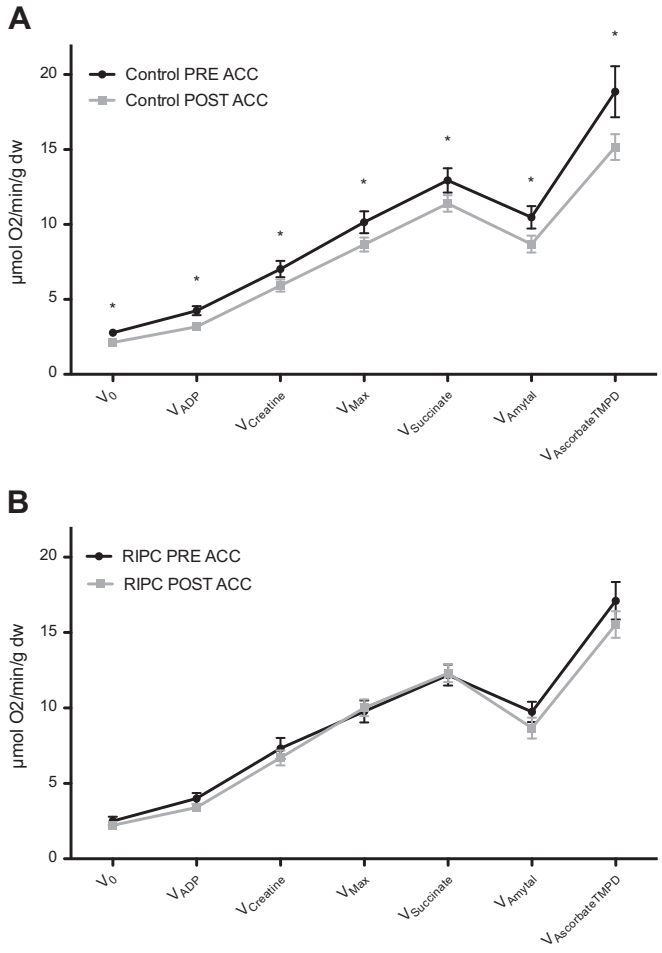

Fig. 4. Mitochondrial respiration rates pre- vs. post-ACC within (A) control group (n = 30) and (B) RIPC (n = 30). V_0, basal respiration; V_{ADP}, subsaturating ADP (0.1 mmol/L); $V_{creatine}$, 20 mmol/L creatine; V_{max}, maximal respiration (2 mmol/L ADP); $V_{succinate}$, 10 mmol/L succinate (Complex-II substrate); V_{amytal}, 1 mmol/L amytal inhibits Complex-I; $V_{ascorbateTMPD}$, 0.5 mmol/L ascorbate and 0.5 mmol/L TMPD stimulate Complex-IV. Data are displayed as mean ± SEM. *p < 0.05.

We observed significantly lower AUC for cTnT and CK-MB only in non-diabetic patients (vs. controls) receiving RIPC (Supplemental Fig. 1). Although the number of individuals with diabetes is too small in our study to draw any conclusions with regard to effects of RIPC in patients with diabetes, a previous study reported that diabetic patients do not benefit from the preconditioning effect of prodromal angina 24 h prior myocardial infarction [19]. Interestingly, experimental evidence suggested that diabetes negatively interferes with preconditioning due to a reduced ability of diabetic hearts to enhance activation of Akt upon preconditioning [32].

Most studies on RIPC adopted postoperative circulating levels of cardiac biomarkers as the only endpoint to infer cardioprotection [1,33,34]. This created a precipitated sense that similar levels of cardiac biomarkers between control and RIPC mean that the intervention promoted no cardioprotection at all. Such assumption led studies to conclude that RIPC was ineffective [33] or even "disappointing" [35]. On the other hand, a recent RIPC clinical trial reported enhanced myocardial salvage after 30 days and improved long-term outcomes in RIPC group, even though no differences were observed in postoperative troponin release [36,37]. Therefore, circulating "cardiac biomarkers" should not be the only indexes to assess cardioprotection, especially when ventricular biopsies permit assessment of biological phenomena in myocardial tissue.

Interfering effects of anesthetics such as isoflurane [38], opioids [39] and propofol [34] are frequently debated when it comes to assessing RIPC effects on serum cardiac markers, but a consensus onto what extent they interfere with RIPC has not been reached. RIPC has been demonstrated to reduce postoperative levels of circulating cardiac markers both with [34] and without isoflurane [1], while other studies found no such effect with [40] or without isoflurane [35], or with propofol [34]. In our study, the anesthetic regimen included isoflurane, propofol and fentanyl for all patients. As the anesthetic protocol was equivalent for both groups, this would not affect the differences observed between control and RIPC.

We believe that our results are important from a clinical perspective because they provide evidence that intracellular mechanisms previously demonstrated in experimental models are also relevant to humans. This study supports the hypothesis that mitochondrial function may play an important role in effects of RIPC in the human left ventricle, and that early activation of Akt may be essential to evoke cardioprotection. These results encourage the pursuit for selective Akt activators as potential pharmacological agents aiming to evoke cardioprotection in clinical settings.

4.1. Limitations

Despite randomization, the control group included a statistically higher number of patients taking calcium antagonists. We could however not find any report indicating that this should affect the influence of RIPC on mitochondrial respiration or Akt activation. Additionally, mitochondrial respiration and Akt activation did not differ between patients with and without calcium antagonists preoperatively.

Our study did not include a sufficient number of patients with diabetes to make conclusions regarding the effects of RIPC in diabetics. Further studies are needed to establish the effect of RIPC in this patient group.

Table 2
Mitochondrial respiratory parameters.

	Control (N = 30)			RIPC (N = 30)		
	Pre-ACC	Post-ACC	p-Value	Pre-ACC	Post-ACC	p-Value
V_{amytal}/V_{max}	1.02 ± 0.4	0.98 ± 0.2	0.72	0.96 ± 0.2	0.89 ± 0.3	0.35
V_{ADP}/V_{max}	0.46 ± 0.2	0.37 ± 0.1	0.06	0.42 ± 0.2	0.35 ± 0.2	0.11
ACR	4.07 ± 2.0	4.96 ± 2.7	0.16	4.80 ± 3.0	5.70 ± 3.4	0.21
↑RR Cr, %	72 ± 58	85 ± 36	0.21	94 ± 59	101 ± 53	0.62
$^{app}K_m^{(ADP+Cr)}$, µM	57 ± 42	70 ± 56	0.41	44 ± 36	60 ± 56	0.19
$^{app}K_m^{(ADP-Cr)}$, µM	151 ± 95	198 ± 121	0.14	174 ± 92	222 ± 128	0.11

V_{amytal}/V_{max}, ratio quantifying excess respiration of the cytochrome oxidase complex; V_{ADP}/V_{max}, ADP sensitivity ratio; ACR, acceptor control ratio; ↑RR Cr, percent increase in respiration rate after addition of creatine; $^{app}K_m^{(ADP+Cr)}$, apparent Michaelis–Menten constant for ADP in the presence of creatine; $^{app}K_m^{(ADP-Cr)}$ apparent Michaelis–Menten constant for ADP in the absence of creatine.

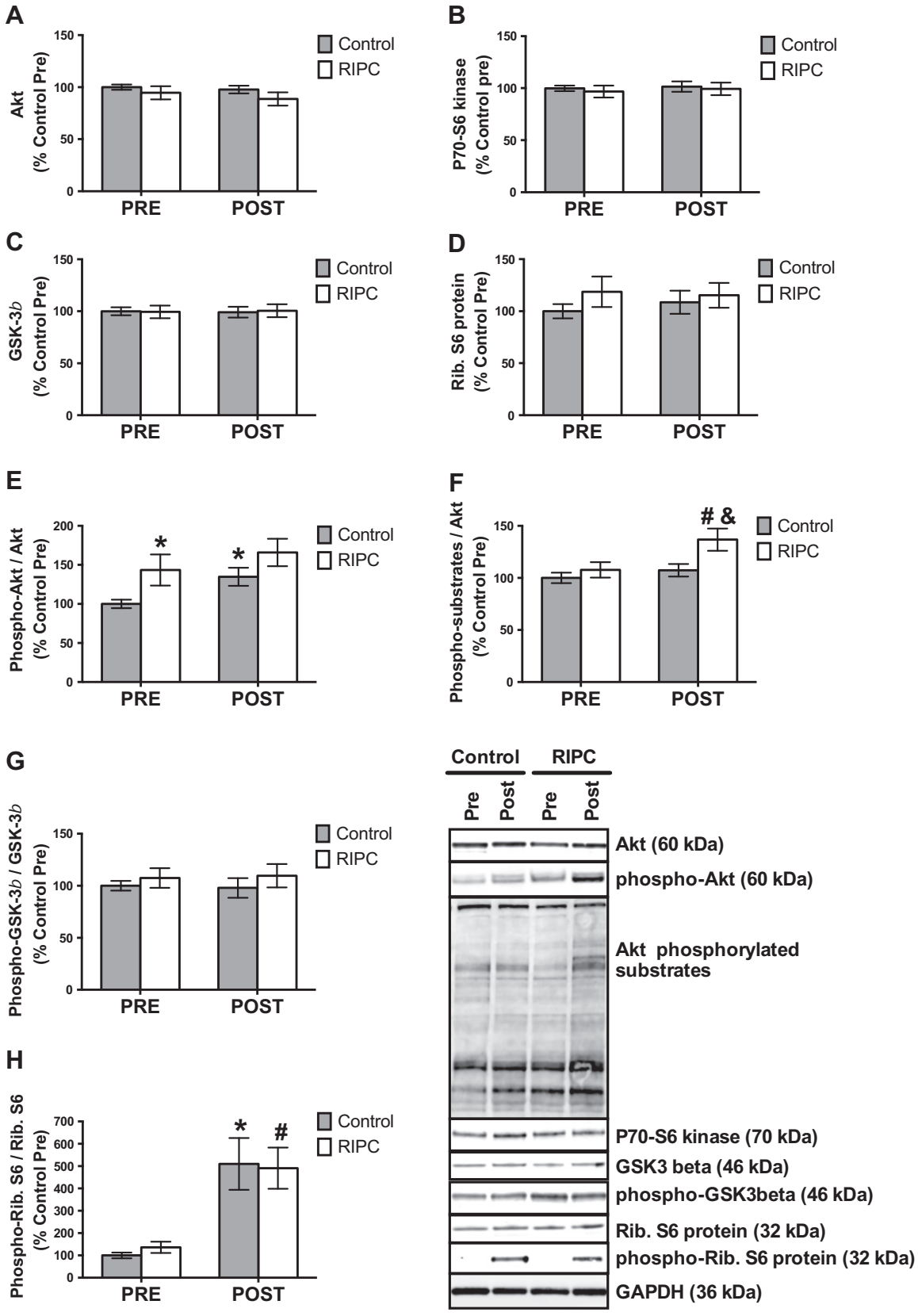

Fig. 5. Protein expression and phosphorylation in control group (n = 24) and RIPC (n = 24). Non-phosphorylated abundance and phosphorylated levels pre-ACC and post-ACC are shown. Original representative bands are presented. Data are presented as mean ± SEM. *p < 0.05 vs. control-PRE; #p < 0.05 vs. RIPC-PRE; &p < 0.05 vs. control-POST.

Due to sample availability, we were unable to perform experiments that required a different sample preparation. For instance, assessment of apoptosis by gold-standard TUNEL assays (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) could have provided additional insight on a relationship between mitochondrial function, Akt activation and cardiomyocyte death.

5. Conclusion

RIPC preserved mitochondrial function and activated the anti-apoptotic protein kinase Akt in the left ventricle of patients undergoing CABG surgery. Our results indicate that RIPC induces alterations in the left ventricular myocardium and provides a novel aspect in the investigation of RIPC in the clinical setting. Further investigations focusing on modulation of mitochondrial function and activation of Akt may provide valuable knowledge in optimizing cardioprotection against ischemia–reperfusion.

Authors contribution

Katrine Slagsvold and Jose Moreira take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. Study concept and design: Slagsvold, Rognmo, Høydal, Wisløff and Wahba. Acquisition of data: Slagsvold and Moreira. Analysis and interpretation of data: Slagsvold, Moreira, Rognmo, Høydal, Bye, Wisløff and Wahba. Drafting of the manuscript: Slagsvold and Moreira. Critical revision of the manuscript for important intellectual content: Slagsvold, Moreira, Rognmo, Høydal, Bye, Wisløff and Wahba. Statistical analysis: Slagsvold and Moreira. Obtained funding: Slagsvold, Wahba and Wisløff. Study supervision: Wahba and Wisløff.

Conflicts of interest

The authors report no relationships that could be construed as a conflict of interest.

Acknowledgments

The authors are particularly grateful to the cardiothoracic surgeons of St. Olav's University Hospital of Trondheim for providing the biopsies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2014.09.206.

References

[1] Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E, et al. Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. Lancet 2007;370:575–9.
[2] Perrelli MG, Pagliaro P, Penna C. Ischemia/reperfusion injury and cardioprotective mechanisms: role of mitochondria and reactive oxygen species. World J Cardiol 2011;3:186–200.
[3] Chen Q, Camara AK, Stowe DF, Hoppel CL, Lesnefsky EJ. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion. Am J Physiol Cell Physiol 2007;292:C137–47.
[4] Uchiyama T, Engelman RM, Maulik N, Das DK. Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. Circulation 2004;109:3042–9.
[5] Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 2007;1767:1007–31.
[6] Thaveau F, Zoll J, Rouyer O, Chafke N, Kretz JG, Piquard F, et al. Ischemic preconditioning specifically restores complexes I and II activities of the mitochondrial respiratory chain in ischemic skeletal muscle. J Vasc Surg 2007;46:541–7 [discussion 7].
[7] Suleiman MS, Halestrap AP, Griffiths EJ. Mitochondria: a target for myocardial protection. Pharmacol Ther 2001;89:29–46.
[8] Neuzil J, Widen C, Gellert N, Swettenham E, Zobalova R, Dong LF, et al. Mitochondria transmit apoptosis signalling in cardiomyocyte-like cells and isolated hearts exposed to experimental ischemia–reperfusion injury. Redox Rep 2007;12:148–62.
[9] Narula J, Pandey P, Arbustini E, Haider N, Narula N, Kolodgie FD, et al. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci U S A 1999;96:8144–9.
[10] Kunuthur SP, Mocanu MM, Hemmings BA, Hausenloy DJ, Yellon DM. The Akt1 isoform is an essential mediator of ischaemic preconditioning. J Cell Mol Med 2012;16:1739–49.
[11] Hausenloy DJ, Iliodromitis EK, Andreadou I, Papalois A, Gritsopoulos G, Anastasiou-Nana M, et al. Investigating the signal transduction pathways underlying remote ischemic conditioning in the porcine heart. Cardiovasc Drugs Ther 2012;26:87–93.
[12] Matsui T, Tao J, del Monte F, Lee KH, Li L, Picard M, et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation 2001;104:330–5.
[13] Rane S, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, et al. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. Circ Res 2009;104:879–86.
[14] Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, et al. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. Circulation 2009;119:2357–66.
[15] Li P, Jiao J, Gao G, Prabhakar BS. Control of mitochondrial activity by miRNAs. J Cell Biochem 2012;113:1104–10.
[16] N'Guessan B, Zoll J, Ribera F, Ponsot E, Lampert E, Ventura-Clapier R, et al. Evaluation of quantitative and qualitative aspects of mitochondrial function in human skeletal and cardiac muscles. Mol Cell Biochem 2004;256–257:267–80.
[17] Kuznetsov AV, Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc 2008;3:965–76.
[18] Kersten JR, Toller WG, Gross ER, Pagel PS, Warltier DC. Diabetes abolishes ischemic preconditioning: role of glucose, insulin, and osmolality. Am J Physiol Heart Circ Physiol 2000;278:H1218–24.
[19] Ishihara M, Inoue I, Kawagoe T, Shimatani Y, Kurisu S, Nishioka K, et al. Diabetes mellitus prevents ischemic preconditioning in patients with a first acute anterior wall myocardial infarction. J Am Coll Cardiol 2001;38:1007–11.
[20] Dzeja PP, Holmuhamedov EL, Ozcan C, Pucar D, Jahangir A, Terzic A. Mitochondria: gateway for cytoprotection. Circ Res 2001;89:744–6.
[21] Ferrari R, Cargnoni A, Ceconi C. Anti-ischaemic effect of ivabradine. Pharmacol Res 2006;53:435–9.
[22] Ventura-Clapier R, Garnier A, Veksler V, Joubert F. Bioenergetics of the failing heart. Biochim Biophys Acta 1813;2011:1360–72.
[23] Yellon DM, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. Lancet 1993;342:276–7.
[24] Stride N, Larsen S, Hey-Mogensen M, Hansen CN, Prats C, Steinbruchel D, et al. Impaired mitochondrial function in chronically ischemic human heart. Am J Physiol Heart Circ Physiol 2013;304:H1407–14.
[25] Lemieux H, Semsroth S, Antretter H, Hofer D, Gnaiger E. Mitochondrial respiratory control and early defects of oxidative phosphorylation in the failing human heart. Int J Biochem Cell Biol 2011;43:1729–38.
[26] Mansour Z, Bouitbir J, Charles AL, Talha S, Kindo M, Pottecher J, et al. Remote and local ischemic preconditioning equivalently protects rat skeletal muscle mitochondrial function during experimental aortic cross-clamping. J Vasc Surg 2012;55:497–505e1.
[27] Miyamoto S, Murphy AN, Brown JH. Akt mediated mitochondrial protection in the heart: metabolic and survival pathways to the rescue. J Bioenerg Biomembr 2009;41:169–80.
[28] Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–41.
[29] Cheng Y, Zhu P, Yang J, Liu X, Dong S, Wang X, et al. Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4. Cardiovasc Res 2010;87:431–9.
[30] Sato T, Marban E. The role of mitochondrial K(ATP) channels in cardioprotection. Basic Res Cardiol 2000;95:285–9.
[31] Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 2008;359:473–81.
[32] Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM. Preconditioning the diabetic heart: the importance of Akt phosphorylation. Diabetes 2005;54:2360–4.
[33] Lomivorotov VV, Shmyrev VA, Nepomnyaschih VA, Ponomarev DN, Knyazkova LG, Lomivorotov VN, et al. Remote ischaemic preconditioning does not protect the heart in patients undergoing coronary artery bypass grafting. Interact Cardiovasc Thorac Surg 2012;15:18–22.
[34] Kottenberg E, Thielmann M, Bergmann L, Heine T, Jakob H, Heusch G, et al. Protection by remote ischemic preconditioning during coronary artery bypass graft surgery with isoflurane but not propofol — a clinical trial. Acta Anaesthesiol Scand 2012;56:30–8.
[35] Rahman IA, Mascaro JG, Steeds RP, Frenneaux MP, Nightingale P, Gosling P, et al. Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? Circulation 2010;122:S53–9.
[36] Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, et al. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet 2010;375:727–34.
[37] Sloth AD, Schmidt MR, Munk K, Kharbanda RK, Redington AN, Schmidt M, et al. Improved long-term clinical outcomes in patients with ST-elevation myocardial infarction undergoing remote ischaemic conditioning as an adjunct to primary percutaneous coronary intervention. Eur Heart J 2014;35:168–75.

[38] Lee MC, Chen CH, Kuo MC, Kang PL, Lo A, Liu K. Isoflurane preconditioning-induced cardio-protection in patients undergoing coronary artery bypass grafting. Eur J Anaesthesiol 2006;23:841–7.
[39] Dragasis S, Bassiakou E, Iacovidou N, Papadimitriou L, Andreas Steen P, Gulati A, et al. The role of opioid receptor agonists in ischemic preconditioning. Eur J Pharmacol 2013;720:401–8.
[40] Lucchinetti E, Bestmann L, Feng J, Freidank H, Clanachan AS, Finegan BA, et al. Remote ischemic preconditioning applied during isoflurane inhalation provides no benefit to the myocardium of patients undergoing on-pump coronary artery bypass graft surgery: lack of synergy or evidence of antagonism in cardioprotection? Anesthesiology 2012;116:296–310.
[41] Nashef SA, Roques F, Sharples LD, Nilsson J, Smith C, Goldstone AR, et al. EuroSCORE II. Eur J Cardiothorac Surg 2012;41:734–44 [discussion 44–5].